

A POSSIBLE ROLE OF NERVE GROWTH FACTOR DURING
NEURAL DIFFERENTIATION AND DURING REGENERATION
OF CENTRAL AND PERIPHERAL NORADRENERGIC NEURONS

BY

BO BJERRE

Lund 1974

From the Institute of Anatomy and Histology,
University of Lund, Lund, Sweden

A POSSIBLE ROLE OF NERVE GROWTH FACTOR
DURING NEURAL DIFFERENTIATION AND DURING
REGENERATION OF CENTRAL AND PERIPHERAL
NORADRENERGIC NEURONS

BY

BO BJERRE

Lund 1974



The present summary is based on the following papers:

Part I

- I. Effect of mouse submaxillary gland and NGF preparations on competent chick ectoderm in vitro, studied by the immunofluorescence method. Wilhelm Roux' Arch. Entwickl.-Mech. Org. 171 (1972) 38-47 (together with L. Nord).
- II. Effects of nerve growth factor on competent chick ectoderm. Experientia 29 (1973) 1018-1019 (together with L. Nord).
- III. An immunofluorescence study of in vitro neural induction and early neuralization of chick blastoderm: Effect of anti-NGF serum. Acta Morphol. Neerl.-Scand. 12 (1974) 25-45.
- IV. The production of catecholamine-containing cells in vitro by young chick embryos: Effects of 'Nerve Growth Factor' (NGF) and its anti-serum. Neurobiology 3 (1973) 140-161 (together with A. Björklund).

Part II

- V. Stimulation of growth of new axonal sprouts from lesioned monoamine neurones in adult rat brain by nerve growth factor. Brain Research 60 (1973) 161-176 (together with A. Björklund and U. Stenevi).

- VI. Effects of localized intracerebral injections of nerve growth factor on the regenerative growth of lesioned central noradrenergic neurones. Brain Research 69 (1974) 217-234 (together with U. Stenevi, A. Björklund and W. Mobley).
- VII. Inhibition of the regenerative growth of central noradrenergic neurons by intracerebrally administered anti-NGF serum. Brain Research 74 (1974) 1-18 (together with A. Björklund and U. Stenevi).
- VIII. A stimulatory effect by nerve growth factor on the regrowth of adrenergic nerve fibres in the mouse peripheral tissues after chemical sympathectomy with 6-hydroxydopamine. Z. Zellforsch. 146 (1973) 15-43 (together with A. Björklund and W. Mobley).
- IX. Axonal regeneration of peripheral adrenergic neurons: Effects of antiserum to nerve growth factor in mouse. Z. Zellforsch. 148 (1974) 441-476 (together with A. Björklund and D.C. Edwards).

In the following text, these papers are referred to by their Roman numerals.

CONTENTS

1.	INTRODUCTION.....	7
1.1	Nerve Growth Factor.....	7
1.2	Neural Induction and Early Differentiation of Nerve Cells.....	13
1.3	Axonal Regeneration.....	15
2.	MATERIAL AND METHODS.....	17
2.1	NGF Preparations.....	17
2.2	Anti-NGF Serum Preparations.....	18
2.3	Culture Technique.....	18
2.4	Operative Procedures in Rats.....	19
2.5	Chemical Sympathectomy in Mice.....	21
2.6	Immunofluorescence Method.....	22
2.7	Histochemical Procedures.....	22
2.8	Noradrenaline Assay.....	23
3.	RESULTS.....	24
3.1	NGF-Induced Neuralization of Competent Ectoderm <u>in vitro</u>	24
3.2	Anti-NGF Serum-Induced Inhibition of Neuralization <u>in vitro</u>	25
3.3	NGF- and Anti-NGF Serum-Induced Effects During the Differentiation <u>in vitro</u> of Catecholamine-Containing Cells.....	26
3.4	NGF-Induced Stimulation of the Regenerative Growth of Lesioned Central Monoamine Neurons.	27
3.5	Anti-NGF Serum-Induced Inhibition of the Regenerative Growth of Lesioned Central Noradrenergic Neurons.....	28
3.6	NGF-Induced Stimulation and Anti-NGF Serum-Induced Inhibition of the Regenerative Growth of Peripheral Noradrenergic Neurons...	29

4.	DISCUSSION.....	30
4.1	A Possible Role for NGF During the Early Differentiation of Nerve Cells in Chick Embryos.....	30
4.2	A Possible Role for NGF During Axonal Regeneration of Central and Peripheral Noradrenergic Neurons.....	36
5.	GENERAL DISCUSSION.....	43
6.	REFERENCES.....	46

1. INTRODUCTION

1.1 Nerve Growth Factor (NGF)

In 1948, Bueker reported that mouse sarcoma 180 grafted into the body wall of 3-day old chick embryos resulted in a growth response from sensory ganglia adjacent to the tumour. When repeating Bueker's experiment, Levi-Montalcini and Hamburger (1951) and Levi-Montalcini (1952) obtained evidence that mouse sarcomas (180 and 37) released a diffusible factor - later known as NGF - into the circulation of the host, and that this factor enhanced the growth of both sensory and sympathetic nerve cells. For further analysis of the characteristics of this growth factor and of the growth response elicited by it, an in vitro technique was devised (Levi-Montalcini et al., 1954). By this technique also a large number of fragments and extracts of tissues were tested for the presence of the nerve growth-promoting factor.

Today, about 20 years after the detection of NGF, there is a voluminous literature concerning this factor; thus only some major outlines can be discussed here.

When present in adequate concentrations, NGF produces a "fibrillar halo" of outgrowing fibres from developing sensory and sympathetic ganglia in vitro. Injections of NGF into the yolk sac of 6- to 10-day old chick embryos resulted in a profuse overgrowth of cutaneous sensory nerve fibres (Levi-Montalcini, 1958) and a marked overgrowth of sympathetic nerve fibres in several visceral organs (Levi-Montalcini and Cohen, 1956; for review, see Levi-Montalcini and Angeletti, 1968). Simultaneously a marked enlargement of sensory and sympathetic ganglia could be observed. In newborn

mammals NGF injections call forth an enlargement of sympathetic ganglia and a marked, adrenergic hyperinnervation of several organs (cf. Olson, 1967), whereas no clear effects are observed in the sensory ganglia of the animals (for reviews, see Levi-Montalcini and Angeletti, 1968; Levi-Montalcini et al., 1972). The outgrowth of fibres elicited by NGF from sensory and sympathetic ganglia in vitro is accompanied by metabolic changes such as an increased synthesis of RNA, proteins, and lipids, and a stimulated glucose utilization (cf. Angeletti et al., 1964a, b; 1965). However, there is no compelling evidence showing that nerve growth factor has a direct effect on RNA synthesis, and Partlow and Larrabee (1971) found that the effects of NGF on RNA synthesis are, at least partly, independent of those on the fibre outgrowth (for review, see Larrabee, 1972). On the other hand, NGF in vitro appears to stimulate de novo synthesis of neurotubule subunit protein, and this effect may precede neurite extension (see Hier et al., 1972; also cf. Yamada and Wessels, 1971). At the ultrastructural level several impressive changes follow the NGF stimulation of developing sensory ganglion cells in vitro (Levi-Montalcini et al., 1968), and of developing and adult sympathetic ganglion cells in vivo (Angeletti et al., 1971a).

The presence of NGF in a wide variety of vertebrate species and in a variety of organs, tissues, and body fluids of mammals, e.g., mouse or rat, is well documented (see, for instance, Winick and Greenberg, 1965; Johnson et al., 1971; Hendry, 1972; Hendry et al., 1972; Banks et al., 1973; for review, see Levi-Montalcini and Angeletti, 1968). For somewhat obscure reasons, the submaxillary glands of the male mouse stand out as the most potent source of NGF, whereas no NGF activity

was detected in the submaxillary glands of other mammalian species (Winick and Greenberg, 1965; Banks et al., 1973). It seems probable that there is an in situ production of NGF in the mouse submaxillary glands (Burdman and Goldstein, 1965; Levi-Montalcini and Angeletti, 1968), and that these glands play an active role in maintaining the NGF level, not only in the blood (cf. Weis and Bueker, 1966), but also in most tissues of adult mice (Hendry and Iversen, 1973). Also other tissues in the mouse seem able to synthesise NGF. Thus, subsequent to the marked reduction in the level of circulating NGF after removal of the male mouse submaxillary glands, a fairly rapid recovery occurs, and by 60 days after salivectomy, normal NGF levels are reached, in spite of any significant regeneration of the glands (Hendry and Iversen, 1973). Although there is no definite proof that the submaxillary glands are essential or even necessary for the NGF production in mouse, it none the less seems that much of the synthesis of NGF is normally accomplished by these glands. The submaxillary glands also appear to have a regulatory effect on NGF synthesis of other tissues, which, on the other hand, after a certain period of adaptation following salivectomy, become capable of taking over the synthesis of NGF normally accomplished by the submaxillary glands (Hendry and Iversen, 1973). In mammalian species other than mouse, it could well be that NGF, including that present in the serum, is normally produced everywhere in the tissues (cf. Levi-Montalcini and Angeletti, 1972; Banks et al., 1973).

Over the past few years several data have been presented about the character of the NGF proteins, particularly about those proteins purified from the mouse submaxillary glands, and it has become apparent

that NGF activity is not uniquely associated with a single protein species. By purification from the mouse submaxillary glands two major protein classes with NGF activity can be identified (for review, see Pérez-Polo et al., 1972): a high molecular weight complex protein (designated 7 S NGF), existing in neutral pH homogenates of the glands, and the smaller, basic proteins, which can be isolated from the high molecular weight species after exposure to mild acid or alkaline pH (Varon et al., 1967b). The 7 S NGF complex can thus be dissociated into three types of subunit of which one, the so-called β -subunit, accounts for the nerve growth-promoting activity of this complex. This β -subunit closely resembles the so-called 2.5 S NGF species (cf. Pérez-Polo et al., 1972), which was isolated by Bocchini and Angeletti (1969) from the mouse submaxillary glands. However, the recovery of NGF activity appears much higher for the 2.5 S NGF than for the β -subunit. Recently, the complete amino-acid sequence of the 2.5 S NGF was determined, and the protein was shown to be composed of two identical subunits, each with 118 amino acids (Angeletti and Bradshaw, 1971). According to Pérez-Polo et al. (1972) there is ample evidence that 7 S NGF is a naturally occurring protein in the mouse submaxillary gland.

By injecting an antiserum to NGF into newborn mammals, drastic effects on the sympathetic nervous system are obtained. These were first described by Levi-Montalcini and Booker (1960) and Levi-Montalcini and Cohen (1960). In the newborn animal, the antiserum thus produced a selective and permanent destruction of most of the sympathetic nerve cells, and this phenomenon, called immunosympathectomy, has since been extensively documented (for reviews, see Levi-Montalcini and Angeletti, 1966; Levi-Montalcini, 1972). Electron-microscopic

studies have demonstrated the very rapid effects elicited by the antiserum on sympathetic ganglion cells of newborn mice in vivo and in vitro (Sabatini et al., 1965). Recently, Angeletti et al. (1971b) reported that repeated injections of anti-NGF serum also severs the sympathetic chain ganglia and the sympathetic function in adult animals, but in contrast to the developing system, the adult neurons largely survived the treatment, the impairment being only temporary. Thus, within a few weeks after the end of the treatment, a gradual and progressive recovery of the function ensued.

Some more or less established ideas and hypotheses about the function and role of NGF have provided a challenge; thus they have to some extent served as starting points for the studies presented here.

1. It has previously been claimed that the target nerve cells become receptive to NGF only after they have acquired unmistakable marks of differentiation (cf. Levi-Montalcini and Angeletti, 1968). Those studies presented in Part I were made to investigate the validity of this statement. Thus the possibility of a neuralizing influence of NGF on competent ectoderm (Papers I and II), the possibility for a role of NGF during neural induction and early neuralization (Paper III), and the possibility for a function of NGF during differentiation of catecholamine (CA)-containing cells (Paper IV) were studied.

2. For several years it has been tacitly concluded that NGF acts only on the neural crest derived sympathetic and sensory neurons (see, for instance, Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968). Thus, no clear effects of NGF or anti-NGF serum had been observed in the central nervous system (CNS).

However, Hillman and Sheikh (1968) reported that NGF in vitro promotes the development of processes from isolated vestibular neurons of young or adult rabbits. With reference to these findings, it thus rather seems that the definition of an NGF target tissue is still an open matter; this is further supported by the fact that the in vitro uptake of NGF does not appear to be restricted to tissues known as NGF target tissues (Burnham and Varon, 1973). The studies presented in Papers V-VII were carried out to investigate whether also central monoamine-containing neurons could be regarded as NGF target cells.

3. The studies in Papers V-VII were also made to find out whether NGF and anti-NGF serum treatments would influence the process of regeneration of such neurons in the adult animal. In parallel, studies were also performed to clarify whether NGF could perhaps play a role during the regeneration of peripheral sympathetic neurons in vivo (Papers VIII and IX). Although adult sympathetic neurons appear to be sensitive to NGF during axonal regeneration in vitro (cf. Silberstein et al., 1971; Johnson et al., 1972), no convincing data had previously been presented suggesting that NGF would be of importance during regeneration of any neurons in vivo (cf. Scott and Liu, 1964; Saunders, 1972).

Below, some outlines concerning neural induction, early differentiation of nerve cells, and axonal regeneration are briefly introduced.

1.2 Neural Induction and Early Differentiation of Nerve Cells

In vertebrates, ectodermal cells are induced to form the neural plate — the primordium of the neural system — by an influence from the underlying mesoderm. The nature of the neural inductive influence, which is apparently transferred from the mesoderm into the overlying ectoderm, has not been finally determined. However, it seems to be a chemical substance (probably a protein), as an inductive process can occur across a millipore membrane not penetrable by cells (see, for instance, Gallera et al., 1968; and, for review, Saxén and Toivonen, 1962). The subsequent segregation of the neural plate into various brain regions is most probably the result of at least two successive inductive influences emanating from the mesodermal substratum (for review, see Saxén and Toivonen, 1962). It is also possible that, for the early control of growth and other developmental processes, further interactions between the chordamesoderm and the neural tube is necessary, at least for some time (cf. Källén, 1965, 1968). In agreement with this idea Toivonen and Saxén (1968) demonstrated that the outcome of the neural differentiation in explants depended on the relative amounts of mesodermal cells present surrounding induced neural cells.

The cells in the neural plate — the first visible sign of neural differentiation — are still morphologically undifferentiated, but the cuboidal ectodermal cell layer has now been transformed into a pseudo-stratified epithelium, which later will be the neural epithelium. The cells in this neural epithelium represent the origin of neurons and most neuroglial cells. During neural tube formation, the cells are further elongated and the

pseudo-stratification becomes more pronounced. Within the neural epithelium, single cells later mature into neurons and send out neurites, and during further development cells detach from the neural epithelium and migrate into the marginal layer to form a mantle zone of differentiating neurons. It is not known, however, when the differential gene expression occurring in neuroepithelium cells results in divergent cytodifferentiation of their progeny.

After the primary induction, there is a considerable time lag before the onset of a visible morphological differentiation of the neural tube cells can be demonstrated. With silver staining, neuroblasts with processes can be seen in the chick embryo already at about stage 11 (stage according to Hamburger and Hamilton, 1951), i.e. after hardly 2 days of incubation (Lyser, 1966). The first cells in the neural tube that show morphological differentiation are the motor neuroblasts. By use of the immunofluorescence method, neural antigens were first detected in chick embryos at stages 14-15, i.e. almost simultaneously with the beginning of a visible morphological differentiation of the neural tube (Nord, 1969). It was also shown that this method could be used for the detection of neural antigens in in vitro explants from young chick embryos, despite no visible morphological differentiation into neural structures being demonstrated (Nord, 1969). It should be emphasised, however, that the presence of such antigens does not prove the existence of differentiated neural cells; it only indicates that one aspect of neural differentiation has been fulfilled, and when used in this connection, the term neuralization only refers to that phenomenon. None the less, the presence of neural antigens seems to reveal a development along the neural pathway.

At the margin of the neural plate, neural crest tissue develops. The crest cells undergo migration and differentiation into a large number of tissue types including sensory and sympathetic neurons. The histochemical fluorescence method of Falck and Hillarp (Falck, 1962; Falck et al., 1962) makes it possible to identify the sympathetic neurons at a stage when they are able to store endogenous catecholamines (CA). Thus CA are first demonstrated histochemically in ovo in the sympathetic ganglia of chick embryos at about 3 1/2 days of incubation (Enemar et al., 1965), i.e. shortly after the sympathoblasts have reached the sites where the ganglia develop. Factors that might promote the differentiation of trunk neural crest cells into sympathoblasts have recently been investigated, and it was found probable that chick crest cells must interact with both ventral neural tube and somite mesenchyme in order to differentiate into sympathoblasts (Cohen, 1972; Norr, 1973).

1.3 Axonal Regeneration

One mode of reawakening the morphogenetic processes at an advanced stage of maturation is by means of a partial destruction of the system that has evolved as a result of the previous development. With regard to axonal regeneration, this can be understood as the attempt of a neuron to re-establish axonal connections severed by a lesion. But the capacity and possibility for a successful axonal regeneration varies markedly between different groups of animals and apparently also between different parts of the nervous system. Whereas regeneration in the peripheral nervous system of mammals usually leads to the re-formation of at least part of the severed connec-

tions, regeneration in the brain and the spinal cord is generally considered to be very limited, and if regenerative attempts are made, they will be abortive and no re-establishment of the original connections will occur (Cajal, 1928; for reviews, see Windle, 1955; Clemente, 1964). Such an apparent lack of regeneration in the CNS does not necessarily mean an inability of the central neurons to regenerate, but it could be that the neurons possess an entirely adequate regenerative capacity which in the damaged brain tissue would be inadequately expressed (cf. Björklund et al., 1974). Thus recent studies have demonstrated that certain central monoamine-containing neurons are able to "reinnervate" a denervated tissue - transplanted to the brain or the spinal cord - in a way quite comparable to that of peripheral sympathetic neurons (Björklund and Stenevi, 1971; Björklund et al., 1971b). Moreover, Olson and Seiger (1972) observed that after transplantation of pieces of tissue from fetal and early postnatal rat brain into the anterior chamber of the eye, the monoamine neurons within the transplant grew out to partly "reinnervate" the sympathetically denervated host iris.

The introduction of a number of neurotoxic pharmacology has offered new possibilities for further studies of the process of regeneration of both central and peripheral monoamine-containing neurons. In studies presented here, 6-hydroxydopamine (6-OH-DA) was used to cause degeneration of peripheral adrenergic axonal networks. The high capacity of peripheral adrenergic nerves to regenerate can thus be demonstrated also in animals subjected to chemical sympathectomy by this drug (Haeusler et al., 1969; Tranzer and Richards, 1971; de Champlain, 1971; Jonsson and Sachs, 1972).

2. MATERIAL AND METHODS

2.1 NGF Preparations

Three separate preparations of NGF proteins, one high molecular weight form and two low molecular weight forms, all purified from male mouse submaxillary glands, were used in the present investigations. The high molecular weight form, 7 S NGF, was prepared according to Varon et al. (1967a). Several different batches of the 7 S NGF preparation were used during the course of the studies¹⁾. One of the low molecular weight forms of NGF used was the β -subunit, produced by dissociation of the 7 S species of the NGF protein according to Varon et al. (1967b) and Smith et al. (1968)²⁾. The second low molecular weight form of NGF, 2.5 S NGF, was prepared according to Bocchini and Angeletti (1969)³⁾. Before use, all NGF preparations were tested for specific activity by the tissue culture method according to Levi-Montalcini et al. (1954). The activity of the NGF preparations thus was expressed in biological units (BU); one BU/ml being arbitrarily defined as the NGF concentration in an assay system showing a maximum (4+) fibre response (cf. Varon et al., 1967a).

1) Most of these batches were kindly supplied by Dr. William Mobley, Stanford University, U.S.A.

2) Obtained from Wellcome Research Laboratories, Beckenham, England.

3) Generous gift from Dr. Rita Levi-Montalcini, Rome, Italy.

2.2 Anti-NGF Serum Preparations

NGF purified from male mouse submaxillary glands was used either to immunize a horse or a rabbit. Horse anti-NGF serum was obtained after injections either of the 7 S NGF²⁾ or of the β -subunit⁴⁾. Rabbit anti-NGF serum was obtained after injections of the 2.5 S NGF³⁾. Only one of the batches of antiserum used was tested for activity in vitro (Paper IX). Complement was always inactivated by heating the sera at 56°C for 30 minutes before use. When tested for specificity, the antiserum to 7 S NGF gave several precipitation lines, whereas the antiserum to 2.5 S NGF gave only a single line when reacted with a crude salivary gland extract. As control sera normal horse and normal rabbit sera were used and also hyperimmune sera other than the anti-NGF sera.

2.3 Culture Technique (Papers I-IV)

Fertile eggs (White Leghorn) were incubated at 39°C. At the desired stage, the embryos were removed from the eggs, placed in sterile phosphate buffered saline (pH 7.1), and staged according to Hamburger and Hamilton (1951). The desired region of the embryo was dissected out with metal needles. The explant — in some cases also together with a piece of tissue acting as an inductor (Papers I and III) — was wrapped in a piece of vitelline membrane. The vitelline membrane with its content, and if used, an underlying inductor (Paper III), was then

4) Kindly supplied by Dr. D.C. Edwards, Beckenham, England.

put on a millipore filter strip. This was placed on a piece of gel-foam (Spongostan^(R), Ferrosan) previously placed in a Leighton flask containing 1 ml culture medium. The culture medium consisted of 3 parts human serum, 3 parts 50% chick embryo extract, and 7 parts Tyrode. The Leighton flasks were incubated at 37°C for 3-10 days before the cultures were prepared for examination. At various points and for varying lengths of time during the culture period, NGF, anti-NGF serum, or control serum preparations were added to the culture medium. Preincubations of explanted tissues in anti-NGF and control serum preparations were also performed in some sets of experiments (Papers III and IV).

2.4 Operative Procedures in Rats (Papers V-VII)

Adult female rats (180-200 g) of the Sprague-Dawley strain were used.

Transplantations. These were performed under general barbiturate anaesthesia (Brietal, Lilly, 40 mg/kg) as autologous iris transplantations according to the technique originally described by Björklund and Stenevi (1971) and later, as improved, by Stenevi et al. (Paper VI). One eye was removed and the iris and the ciliary body were dissected out. With the head of the rat secured in a stereotaxic apparatus, a midline incision of the skin was made. By a small hole drilled in the right parietal bone close to the midline, the dura was carefully opened. With a glass tube - later replaced by a thin, flat rod (cf. Paper VI) - the iris was lowered by free hand into the diencephalon. The iris transplant was placed in such a position, that the lesion caused

by the transplantation transected the major ascending catecholamine pathways, i.e. the medial forebrain bundle (MFB) and the dorsal catecholamine bundle (DCB) (cf. Björklund et al., 1971a; Ungerstedt, 1971).

Intraventricular injections (Papers V and VII). These were made stereotaxically into the lateral ventricle. The desired amount of NGF was injected in a volume of 20 μ l; the controls were given the same volume of saline. Undiluted anti-NGF or control serum was injected in a volume of 25 μ l. Single injections of NGF in doses varying between 20 and 20,000 BU were given at the time of transplantation. A single injection of 2,000 BU of NGF was also given 2 days before the transplantation, 2 days after, or 4 days after. In one set of experiments repeated injections of NGF were given. The NGF-treated and the corresponding control animals were killed 5 or 7 days after transplantation. Anti-NGF serum was administered either as a single injection 7 days after transplantation or as 7 daily injections, starting on the day of transplantation, and all animals, including the corresponding controls, were killed 2 weeks after transplantation.

Intracerebral injections (Papers VI and VII). These were made stereotaxically at three different sites close to the ascending noradrenergic neuron system in the locus coeruleus. Each injection consisted of 2 microlitres of NGF or saline and undiluted anti-NGF or control serum. NGF was given in doses of 2, 20, or 200 BU at the time of transplantation, and in a dose of 20 BU 2 days before, 2 days after, or 4 days after transplantation. The animals were killed 5, 7, or 9 days after transplantation. Anti-NGF serum was given either as a single

injection at the time of transplantation or 4 days after, or as two injections: one at the time of transplantation, and the other 2 weeks later. The anti-NGF serum-treated animals and the corresponding controls were killed 2 or 4 weeks after transplantation.

Preincubations (Papers VI and VII). Before being used for transplantation, irises were incubated at room temperature for 15 minutes in a phosphate buffered solution (pH 7.4) of NGF (containing 100,000 BU/ml), with corresponding controls in saline; or incubated in undiluted rabbit anti-NGF serum, with corresponding controls in normal rabbit or rabbit anti-mouse thymocyte serum. The NGF-treated and the corresponding control animals were killed 7 days after transplantation and the anti-NGF serum-treated animals, including the corresponding controls, 2 weeks after transplantation.

Cervical sympathectomy (Papers V and VII). This was performed by bilateral removal of the superior cervical ganglia before the transplantation.

2.5 Chemical Sympathectomy in Mice (Papers VIII and IX)

4-week old female and 5-week old male albino mice (N.M.R.I. strain) were given one intravenous injection of 60 or 220 mg/kg body weight of 6-OH-DA (AB Hässle), dissolved in saline containing 0.2 mg/ml ascorbic acid. Next day they received one dose of NGF (1,000, 3,000, or 4,000 BU/g) or anti-NGF serum (0.1 ml/g), usually subcutaneously. Control animals were given saline or normal serum similarly. In most series of experiments, the

NGF-treatment was continued; daily injections of NGF were thus given for another 2 or 5 days, and in one set of experiments, for a total of 15 days. Controls were given saline according to the same schedule. In the NGF series of experiments, the animals were killed 9 days or 3 weeks, and in the anti-NGF serum series, 5 days, 3 weeks, or 2 months, after the 6-OH-DA treatment. 4-week old female mice given 60 mg/kg and 5-week old male mice given 60 or 220 mg/kg of 6-OH-DA were also investigated one day after the 6-OH-DA treatment. In addition several untreated female and male mice of varying ages were investigated.

2.6 Immunofluorescence Method (Papers I-III)

Antiserum was prepared against the intracranial part of the CNS of chicks killed at hatching day, according to the method described by Nord (1969). To prepare the cultures for the immunofluorescent investigation, two different methods were used: (1) sectioning in a cryostat, (2) manual squashing. The indirect fluorescence method was employed: for the sections, a two-layer technique; for the squashed cultures, a quadruple method. In the first step, control serum or antiserum versus chick CNS was applied; in the last step, a fluorescein isothiocyanate conjugated antiserum (for further details, see Paper I).

2.7 Histochemical Procedures (Papers IV-IX)

When the cultures explanted from chick embryos were prepared for investigation they were first taken out

from the vitelline membrane and transferred to a glass slide (Paper IV). Subsequently, similar to the whole mount preparations of mouse irises and atria (Papers VIII and IX), they were dried in a desiccator over phosphorous pentoxide for at least 3 hours. The dried cultures and also the irises and atria were treated with formaldehyde gas of optimum humidity according to the Falck-Hillarp method and prepared for fluorescence microscopy (for technical details, see Björklund et al., 1972b).

From the rat brain (Papers V-VII) and from the mouse (except the irises and the atria) (Papers VIII and IX), the desired pieces of tissue were rapidly dissected out, frozen in liquid propane, cooled with liquid nitrogen, and freeze dried. The formaldehyde treatment and the preparation for fluorescence microscopy after sectioning were the same for these freeze-dried tissue pieces as for the air dried ones (see above).

Microspectrofluorometry (Paper IV). The specific fluorescence in the formaldehyde-treated cultures, explanted from chick embryos, was characterized by spectral analysis in a modified Leitz microspectrofluorometer (see Björklund et al., 1972b). The characteristic CA spectra were further analysed according to the HCL vapour method of Björklund et al. (1968, 1972a).

2.8 Noradrenaline Assay (Papers VIII and IX)

Endogenous noradrenaline (NA) was determined fluorometrically according to Bertler et al. (1958) and Häg-gendal (1963) in several mouse organs and tissues.

3. RESULTS

3.1 NGF-Induced Neuralization of Competent Ectoderm in vitro (Papers I and II)

By an immunofluorescence method, neural differentiation was identified as neural antigen production in cultures from chick ectoderm taken at stages 3 to 5, i.e. at about 12-22 hours of incubation. When added to the culture medium on the first day of incubation and present throughout the culture period, 100 (but not 10) BU/ml of NGF markedly increased the frequency of neural antigen-producing cultures in series of explants taken from the presumptive epidermal region at stage 4. Three different NGF preparations from the male mouse submaxillary glands, 7 S NGF, the β -subunit, and 2.5 S NGF (in Paper I referred to as own NGF preparation, commercial NGF, and highly purified NGF, respectively) were tested and were found to act similarly on competent ectoderm. In contrast to the submaxillary gland of the female mouse, that of the male mouse, which contains large amounts of NGF, was observed to have a strong neuralizing influence on competent ectoderm. The results of preincubations of the male mouse submaxillary gland in anti-NGF serum made it probable that the content of NGF in this gland was responsible for its effect on the ectoderm (Paper III). The frequency of neural antigen-producing cultures in series of explants taken from competent ectoderm at stage 4 was seen to gradually increase when NGF added to the culture medium on the first day of incubation was present for successively longer periods of the culture. It was also observed that different regions of the ectoderm through stages 3 to 4 showed slight differences

in tendency for autoneuralization; for instance, the presumptive neural plate ectoderm was found to have a somewhat higher tendency than the presumptive epidermal ectoderm. Such differences in the capacity of autoneuralization were also reflected after NGF treatment.

3.2 Anti-NGF Serum-Induced Inhibition of Neuralization in vitro (Paper III)

The immunofluorescence technique was again used for identification of neural differentiation. With anti-NGF serum present in the culture medium throughout the culture period, the capacity of the Hensen's node region taken from chick blastoderms at stage 4 (the definitive primitive streak stage) to form neural antigens in vitro was strongly inhibited. Two different anti-NGF serum preparations, one prepared against 7 S NGF (referred to as horse anti-NGF serum), the other against 2.5 S NGF (referred to as rabbit anti-NGF serum), gave similar results. The neuralization of Hensen's node region taken at stage 5 (the head-process stage), i.e. at a stage when early neuralization has already started in ovo, was less impaired by anti-NGF serum. No inhibitory effects were found on explanted pieces of the neural folds taken at stage 8 (4-somite stage). The effect of anti-NGF serum on the development of neural antigens from competent ectoderm combined with a heterologous inducer (guinea-pig liver) was also studied. The obtained results suggest that the inhibitory influence of anti-NGF serum is most pronounced during the early stages of neuralization. No evidence was found that NGF (or an NGF-like substance) is of importance for the neural inductive capacity of the alcohol-killed Hensen's node.

3.3 NGF- and Anti-NGF Serum-Induced Effects During the Differentiation in vitro of Catecholamine- Containing Cells (Paper IV)

The presence of cell-bound CA was taken as a main criterion for the differentiation of adrenergic neuroblasts. Such CA were demonstrated by the histochemical fluorescence method of Falck and Hillarp. CA-containing cells had previously been shown to develop, in vitro, in explants made from both the trunk and the cranial neural level of chick embryos at stages 5 to 15 (Bjerre, 1973). The CA-containing cells formed in the trunk level explants were supposed to be sympathoblasts, whereas the cells formed in the cranial level explants were suggested to be identical with sympathoblasts or sympathoblast-like cells (Bjerre, 1973). Two different NGF preparations, 7 S NGF and 2.5 S NGF, were shown to act similarly by stimulating the production of CA-containing cells in explants from young chick embryos. A stimulatory effect was observed when NGF was present in the culture medium throughout the 8-day culture period in the amount of 40-200, but not 4 BU/ml. However, with NGF present in the culture medium for only a short period, the first 2 days of incubation, no increase in the formation of CA-containing cells was observed. At NGF treatment an increased number of CA-containing cells appeared in those series of explants made from both the trunk and the cranial neural levels. It seemed, however, as though the NGF-induced effect was most pronounced in explants taken from younger embryos and that the effect would perhaps gradually disappear at an increasing age of the embryos, i.e. in parallel with the neural crest cells becoming progressively more restricted in their developmental potential (cf. Weston, 1970). Results were also

obtained suggesting that anti-NGF serum exerts an inhibitory effect on the formation of CA-containing cells in explants taken from young embryos (stages 5 and 6) and that this inhibitory effect would disappear at later stages. The results of the series of experiments with preincubations in anti-NGF serum could possibly indicate that NGF (or an NGF-like substance) is already present — and of importance for the further development of presumptive CA-containing cells — in embryos younger than stage 8.

3.4 NGF-Induced Stimulation of the Regenerative Growth of Lesioned Central Monoamine Neurons (Papers V and VI)

The growth of lesioned ascending noradrenaline (NA), dopamine (DA), and indolamine (IA) neuron systems into a denervated iris, transplanted to the caudal diencephalon, was studied. By intraventricular injections, a stimulatory effect of NGF (7 S NGF, the β -subunit, or 2.5 S NGF) on the growth of new axonal sprouts from all the three types of neurons was revealed and was observed as an increased ingrowth of newly formed fibres in the transplant tissue. The effects of injections locally into the brain tissue close to the cell bodies and axons of the locus coeruleus neuron system suggested that the NGF sensitivity is not restricted to any specific part of these NA neurons. Furthermore, experiments with iris transplants incubated in an NGF solution before the transplantation pointed to a role for the NGF contained in the iris in the process of "reinnervation" of the transplant by central noradrenergic neurons. They also suggested that the DA- and IA-containing neurons in the

MFB system are less sensitive to NGF than the NA-containing neurons running in the DCB. Injections of NGF at the time of transplantation and axonal lesioning were shown to be obviously more effective in stimulating the regenerative growth than were injections given before or after that time. Moreover, the NGF-induced growth response was shown to be clearly dose-dependent. No evidence was obtained to suggest that NGF altered the time sequence of events in the regeneration process of the central neurons.

3.5 Anti-NGF Serum-Induced Inhibition of the Regenerative Growth of Lesioned Central Noradrenergic Neurons (Paper VII)

Again the growth into an iris transplant from lesioned NA, DA, and IA neuron systems was studied. As intraventricular injections of anti-NGF serum — although repeated daily for a week — were not found to affect the regenerative growth from these neuron systems, intracerebral injections of the antiserum were exclusively used. Such injections of anti-NGF serum were placed at different sites close to the ascending NA axons from the locus coeruleus, and they resulted in a marked reduction in the regrowth of new axonal sprouts from these axons into the denervated transplant tissue. When incubating the iris transplant in anti-NGF serum before the transplantation, a similar inhibition was observed on the regrowth of these NA axons. Neither with the local injections of antiserum close to the locus coeruleus neuron system nor with the preincubations of transplants in anti-NGF serum were any clear-cut effects observed on the DA and IA neurons in the adjacent MFB system.

3.6 NGF-Induced Stimulation and Anti-NGF Serum-
Induced Inhibition of the Regenerative Growth
of Peripheral Noradrenergic Neurons (Papers
VIII and IX)

Regeneration of fully developed NA neurons was studied in mouse peripheral tissues after chemical axotomy induced by 6-OH-DA. Subsequent to the axotomy NGF was administered either as a single injection or as repeated injections, and anti-NGF serum only as a single injection. The effects of these treatments were evaluated by fluorescence histochemistry and by determinations of the endogenous NA content of several tissues. As observed in the fluorescence microscope, NGF strongly stimulated the regrowth process in most peripheral tissues, probably by causing an increased outgrowth of new axonal sprouts from the proximal ends of the lesioned axons, an accelerated growth of the sprouting fibres, and an accelerated restoration of the intra-axonal NA concentration towards normal in the regenerating axons. The histochemical findings were supported by the observations of significant NGF-induced increases in the recovery of endogenous NA in several peripheral tissues.

Rapidly following the anti-NGF serum treatment, pronounced effects were observed on the whole NA neurons, including marked atrophic changes of the ganglionic cell bodies and an overall strongly impaired regrowth process from the lesioned axons. However, the effects induced by the antiserum appeared to be only temporary. Thus, 2 months after treatment, the ganglion cell bodies had regained a seemingly normal appearance, and the regeneration in the antiserum-treated animals had largely caught up with that of the control animals. These histochemical findings were paralleled by the NA-values obtained in the various peripheral tissues.

4. DISCUSSION

4.1 A Possible Role for NGF During the Early Differentiation of Nerve Cells in Chick Embryos (Part I: Papers I-IV)

Similar to the amphibian ectoderm at the early gastrula stage (for review, see Saxén and Toivonen, 1962), the whole ectoderm area of the chick embryo at stages 3 to 4 is competent to react to neural inductive stimuli (see, for instance, Waddington, 1932, 1934; Woodside, 1937; Gallera and Ivanov, 1964). In ovo, the neural inductive stimuli to the prospective neuroectoderm emanate from the underlying mesoderm, and Hensen's node has been suggested as an organization centre in the chick blastoderm, comparable to the dorsal blastoporal lip in the amphibian embryo (Wetzel, 1929; Hunt, 1929, 1931; Willier and Rawles, 1931; for review, see Waddington, 1952). In in vitro experiments, the natural primary inducers of the chick embryo — the Hensen's node and the head process — were shown to induce neuralization of competent ectoderm also when killed, either by alcohol or heat (Leikola and McCallion, 1968). Similarly, several other tissues from various species are able to act neuralizing on competent chick ectoderm, and such tissues are referred to as heterologous inducers (see, for instance, Pasternak and McCallion, 1962; Leikola and McCallion, 1967; Rostedt, 1968).

The present in vitro observations of effects of anti-NGF serum on the process of early neuralization of explants (Paper III) and on the early formation of CA-containing cells (Paper IV), have provided some indirect evidence that NGF (or an NGF-like substance) is formed

in the chick embryo at a very early stage of development, i.e. already at about one day of incubation. However, by use of more direct methods for detection, such as bioassay, antibody neutralization, and complement fixation techniques, NGF was first demonstrated in chick embryos on the 4th day of incubation (Winick and Greenberg, 1965). With a sensitive radioimmunoassay (cf. Johnson et al., 1971; Hendry et al., 1972) NGF-antigens could perhaps be detected even before the 4th day, but so far, this has not been thoroughly investigated. However, preliminary attempts have been made to detect NGF-antigens in young chick blastoderms by the radioimmunoassay of Hendry et al. (1972) (Bjerre and Hendry, unpublished observations). Although trace amounts of NGF-antigens were possibly found in chick blastoderms at stage 5 and older (but not in younger) stages the results are too preliminary to add any certain and substantial information about the first detectable appearance of NGF during the ontogenesis of the chick embryo. Therefore the present observations of anti-NGF serum-induced effects during the early development of the chick embryo are so far the only indications that NGF (or an NGF-like substance) might be present before the 4th day of incubation.

In the present studies there were some findings to indicate that, in the chick, NGF could be a substance involved in the process of primary induction as a neural inductive agent: (1) Anti-NGF serum was found to inhibit neural differentiation of Hensen's node region particularly when this was explanted at stage 4, before the primary induction had occurred in ovo (Paper III). Thus the NGF-antibodies could possibly interact with NGF (or an NGF-like substance) present in the inductor and of importance for the inductive

process. (2) Exogenous NGF, either as a content of the male mouse submaxillary gland or as a purified protein, called forth an increased frequency of neuralized cultures in series of explants from competent ectoderm (Papers I and II).

There were on the other hand other data, which obviously contradict the idea of NGF as a substance involved, at least primarily, in the process of primary neural induction. (1) Preincubation of the alcohol-killed Hensen's node in anti-NGF serum apparently did not affect its capacity to induce neuralization of competent ectoderm, suggesting that NGF (or an NGF-like substance) does not serve as the neural inductive stimuli normally emanating from the inductor (Hensen's node) (Paper III). It could perhaps be objected that the inducing capacity of the killed and that of the living Hensen's node are qualitatively different (cf. Leikola and McCallion, 1968), but there is no proof of such a difference. (2) The experiments with combination of competent ectoderm and a heterologous inductor convincingly showed that anti-NGF serum exerts a strong inhibitory effect also on the further process of neuralization taking place after the neural inductive stimuli was removed (Paper III). Although anti-NGF serum in these experiments was found to exert the most pronounced effect during that time when the neural induction occurred, the effect of the NGF-antibodies could not be referred to a blocking of the neural inductive stimuli from the inductor. It thus rather seemed probable that NGF (or an NGF-like substance) was formed as an early result of the neuralization of the competent ectoderm following the induction and that this substance was essential for the early neuralization process. (3) Exogenous NGF enhanced neuralization in vitro even when acting on

competent ectoderm for a fairly short time (1 day), but the outcome of neural differentiation was further increased when NGF could act for a longer period (Paper II). These findings suggested that, although exogenous NGF might have a more immediate, inductive, influence on competent ectoderm its most important mode of action could be to act during the early neuralization process by ensuring the survival and further development of cells already neuralized through autoneuralization in the in vitro milieu.

In view of the observations discussed above (Papers I-III), it seems probable, that, in the chick embryo, endogenous NGF (or an NGF-like substance) plays a role in the early neuralization process, but probably not — at least not primarily — in the normal process of primary induction. Moreover, NGF (or an NGF-like substance) appears to be formed already at a very early stage of the process of neuralization, and during the subsequent development, this substance, at least for a short time, seems to be essential for the differentiation and maintenance of the nerve cells. Exogenous NGF could have a permissive function, ensuring the survival of recently neuralized cells which otherwise would not have survived or developed further. Such a possible supportive mode of action of NGF might be compatible with ideas suggested for the role of exogenous NGF during differentiation of sympathoblast. The experiments of Cohen (1972) and Norr (1973) indicate that chick trunk neural crest cells have to interact with both ventral neural tube and somitic mesenchyme in order to differentiate into sympathoblasts. The differentiation of sympathetic neurons from trunk neural crest cells was probably mediated by three subsequent steps of bilateral tissue interactions: between ventral neural tube, somitic

mesenchyme, and crest cells (Norr, 1973). And, in this connection most interesting, Norr suggested that exogenous NGF, instead of playing a role in the early events of sympathoblast differentiation, might be able to replace ventral tube in its action of ensuring the survival of differentiating sympathetic neurons. This idea agrees with the conclusions of the present study about the effects of exogenous NGF on the production of CA-containing cells in vitro; i.e. NGF did not seem to have any immediate, inductive, but rather a more indirect, supportive, influence on the development of such cells (Paper IV). We found that exogenous NGF could increase the final outcome of differentiated CA-containing cells in explants containing neural crest, neural tube, and somitic mesenchyme tissue together. These findings may further suggest that exogenous NGF is not only able to replace, but also to act additively to, the normal function of the ventral neural tube, i.e. to ensure the differentiation of cells that otherwise would not have survived or developed. However, it cannot be excluded that the stimulatory effect of NGF on the production of sympathoblast in vitro is the result also of modes of action of NGF during the differentiation into sympathoblasts other than merely to mimic the function of the ventral neural tube.

The observations that exogenous NGF in vitro probably plays a role by ensuring the survival and differentiation of neural cells, such as those identified by the present immunofluorescence and histofluorescence methods, do not imply that NGF necessarily serves a similar function in ovo. Moreover, the present experiments have only suggested that NGF (or an NGF-like substance) is perhaps formed as an early result of neuralization following the induction, but they do not give any clear information

about which cell elements or tissues could be NGF sources during the early stages of development. But it seems reasonable to think that the primary sources of NGF are not the neural cells themselves, but rather other adjacent cell elements. This would agree with the suggestion made by Burnham et al. (1972) that non-neuronal cell elements produce NGF (or NGF-like substances) for the support of neuronal elements. It could also be consistent with NGF activity first being found in the axial mesoderm of chick embryos (Bueker et al., 1960; Winick and Greenberg, 1965). Thus, merely as a working hypothesis, it could be suggested that, during the early stages of neuralization and differentiation of nerve cells, NGF (or an NGF-like substance) becomes available to the developing neural cell elements from sources such as glial cell precursors (cf. Burnham et al., 1972) and/or the mesodermal substratum. It has in fact been suggested that the mesodermal substratum exerts a continuous influence, at least for some time, on the growth and developmental processes of the neural tube (Källén, 1965, 1968), and it cannot be excluded that mesodermal substratum tissue was responsible for the function attributed to the ventral neural tube in Norr's experiments on differentiation of sympathoblasts (see above). The effects of the NGF-antibodies in the present in vitro systems might thus be the result of an interference with such sources of endogenous NGF (or an NGF-like substance), normally present in the developing chick embryo. However, according to the present findings, the NGF sources are apparently not available to the antibodies, or essential for the neural cell elements, except for a fairly short stage in the early development. Exogenous NGF, on the other hand, might perhaps act on the developing neural cells as a replacement or supplement

for, at least in the in vitro situation, inadequate NGF support from non-neuronal cell elements.

Finally, it must be emphasised that much more information must be gleaned before the possible role of NGF (or an NGF-like substance) during the early developmental events of neural cell elements can be safely deduced. Thus, at present, mainly hypotheses and mere speculations, as found in the above discussion, can be offered, and the following few very elementary questions that still await answer can be posed: Does the effects of the anti-NGF sera in the present systems really reveal NGF or perhaps another substance otherwise not related to NGF in its function, but only randomly cross-reacting with the antibodies of the serum? If NGF really is formed during the early stage of neuralization following the primary induction, would it be possible to characterize the cell elements or tissues that might serve as NGF sources by, for instance, an immunofluorescence method? Does NGF merely serve a permissive role to ensure the survival and further development of neural cell elements? Why does anti-NGF serum apparently only exert an effect during a very early and short stage of the neural development in the chick?

4.2 A Possible Role for NGF During Axonal Regeneration of Central and Peripheral Noradrenergic Neurons (Part II: Papers V-IX)

The present studies have disclosed effects of NGF and its antiserum on the process of axonal regeneration of fully developed, peripheral and central, NA neurons. They have further pointed out that, in the adult rat brain, monoamine-containing neurons other than NA

neurons, such as DA and IA neurons, also are sensitive to exogenous NGF during regeneration. The observations thus suggest that NGF (or and NGF-like substance) plays a role during axonal regeneration of peripheral and central NA neurons, and perhaps also of central DA and IA neurons. As the effects of NGF and anti-NGF serum on regenerating central neurons have been more intensely studied and are best established for NA neurons, the following discussion will exclusively be based on findings on this type of neurons. Considering the extensive documentation of effects elicited by NGF and its antiserum on embryonic and mature sympathetic neurons and the wide-spread distribution of NGF in tissues and organs, including sympathetic ganglia of adult animals, e.g., mice (cf. Johnson et al., 1971; Hendry, 1972; for review, see Levi-Montalcini and Angeletti, 1968), it seems that there exist prerequisites for a role of NGF (or an NGF-like substance) during regeneration of fully developed sympathetic neurons in vivo. Concerning the CNS, there is only one previous report (Hillman and Sheikh, 1968) to suggest that any central neurons would be sensitive to NGF. But, as 7 S NGF-antigens, which probably have not entered from the blood stream (cf. Angeletti et al., 1972), are detected by radioimmunoassay in the brain tissue, there seem to be prerequisites for a role of NGF also in the CNS, and then, perhaps, for various types of central neurons.

As found in the present studies, NGF and anti-NGF serum treatments seem to induce rather similar effects on the regeneration process of peripheral and of central NA neurons. In both these types of neuron systems, exogenous NGF obviously caused the formation of an increased amount of regenerating sprouts and an acceleration of their growth (Papers V, VI, and VIII). Anti-NGF serum, on the contrary, probably had an inhibitory effect on the

formation and also on the growth of newly-formed sprouting fibres from the lesioned axons of these neurons (Papers VII and IX). Whereas the administration of exogenous NGF also resulted in a restoration of the axonal NA towards normal in the regenerating peripheral NA axons (Paper VIII), such a type of NGF-induced effect was not clearly observed in the central NA neuron system (Papers V and VI). Although more consistently demonstrated in the peripheral neuron system (Paper IX), anti-NGF serum seemed to impair the production of the NA transmitter also in the regenerating central NA neurons (Paper VII). Such similarities between the visible effects on the regeneration process from lesioned peripheral and from lesioned central NA neurons might possibly reflect also similar or perhaps identical modes of action of NGF in the cellular events involved in the reaction of the two types of neurons to injury.

The series of experiments with localized intracerebral injections of NGF and with iris transplants preincubated in NGF seemed to signify that the NGF-induced growth response can be elicited at several levels of the regenerating central NA neurons: at the level of the cell bodies, at the level of the non-terminal axons, and also at the level of the sprouting fibres (Paper VI). Another set of experiments with anti-NGF serum similarly administered, suggested that also NGF-antibodies exert effects at different levels of these central NA neurons (Paper VII). Little is known about the NGF uptake mechanisms of the target neurons, but recent findings of Hendry et al. (1974) on peripheral NA neurons might suggest that NGF is taken up by the surviving non-terminal parts of the lesioned central NA axons similar as it is by lesioned peripheral NA axons. Subsequently, NGF could be transported in a retrograde direction to

the cell bodies to act as a trophic factor (cf. Hendry and Iversen, 1973). The NGF-antibodies, on the other hand, perhaps act primarily through a blocking and a removal of NGF (or an NGF-like substance) normally present in the brain and the introduced target tissue, i.e. the iris being "reinnervated" by the sprouting fibres. Studies are now in progress to clarify whether or not NGF and NGF-antibodies exert effects also at different levels of regenerating peripheral NA neurons, but preliminary observations have not yet given convincing evidence in any direction (Svendgaard, Bjerre, and Björklund, in preparation).

To get some ideas about the mechanism or mechanisms by which NGF and NGF-antibodies affect the process of axonal regeneration of fully developed peripheral and central NA neurons, previous studies of their effects on intact, or in vitro cultured, peripheral NA neurons can be consulted. NGF is known to be essential for the survival of sympathetic ganglion neurons in vitro (Levi-Montalcini and Angeletti, 1968), and Jacobson (1970) and Larrabee (1972) have suggested that a physiological role of NGF during the ontogenetic development could be to promote the survival and growth of neurons that would otherwise have degenerated. The action of NGF-antibodies can then be viewed as removing such a factor necessary for the survival of the neurons, an idea supported by the extensive effects obtained after administration of anti-NGF serum to newborn mammals (for reviews, see Levi-Montalcini and Angeletti, 1966; Levi-Montalcini, 1972). But the present systems provide no evidence that the main action of exogenous NGF or anti-NGF serum during axonal regeneration of the fully developed neurons is to permit or prevent the survival of the lesioned

neurons. The primary effects rather appear as an acceleration and a retardation, respectively, of the sprouting process. In view of the potent anabolic effects of exogenous NGF on developing ganglia in vitro (Angeletti et al., 1964a, b, 1965; Partlow and Larrabee, 1971), it seems reasonable to suppose that its mode of action on the regenerating neuron in vivo would be on the ability of the lesioned neuron to respond to axonal damage, i.e. to form and/or sustain axonal sprouts. This is supported by the findings that, during ontogenesis, the young neurons of sympathetic ganglia appear to become highly dependent on NGF for further growth at the time when they start sprouting axons (Jacobson, 1970). It might also be consistent with the observations of Angeletti et al. (1971b) and by unpublished findings in our own laboratory that, in the adult mouse, the degenerative effects of anti-NGF serum seem to be confined primarily to the axons, and to be mainly of a transient nature. Thus NGF is possibly essential for the maintenance of the axons or axon terminals of NA neurons also in the adult animal.

During the period of axonal outgrowth of developing neurons, there is a great increase in the synthesis of proteins that are exported into the growing axons, and at the ultrastructural level, an increase is observed in, for instance, the number of neurotubules and neurofilaments (Fischer and Jacobson, 1970). Also NGF and anti-NGF serum treatments of developing ganglia evoke very significant alterations in RNA and protein synthesis (Angeletti et al., 1965; Burdman, 1967; Partlow and Larrabee, 1971; Halstead and Larrabee, 1972), and in the cell body content of neurotubules and neurofilaments (see for instance Hier et al., 1972; Roisen and Murphy, 1973; Levi-Montalcini et al., 1968;

Angeletti et al., 1971a, b). In fact, there is much evidence that the neurotubules play an important role in axonal outgrowth. Colchicine treatment — which is known to disrupt the neurotubules — results in a retraction of the outgrowing neurites from embryonic sensory ganglia in vitro (Daniels, 1968; Yamada et al., 1970), a phenomenon that obviously also occurs after removal of NGF from the culture medium (Kumar and Steward, 1970). Moreover, it was found that the effects of NGF on neurite outgrowth in vitro are inhibited by disruption of the neurotubules (Hier et al., 1972). In view of these various observations it would seem possible to explain the effects of exogenous NGF and anti-NGF serum on axonal regeneration on the basis of a role of NGF in the metabolic response of the NA neurons to axonal damage (cf. Björklund et al., 1974). Probably, as an expression of neuronal repair chiefly related to the regrowth of the lesioned axons, a retrograde axonal response is triggered in the neuron cell bodies by axotomy. This anabolic reaction is characterized by increases in RNA and protein synthesis (Brattgård et al., 1957; Watson, 1968) and is often accompanied by a chromatolytic reaction (for further details, see, for instance, Matthews and Raisman, 1972). Moreover, in the regenerating axons, neurofilament proliferation is a prominent feature (Kapeller and Mayor, 1969), which is probably an expression of the regenerative efforts closely related to the sprouting process (cf. Yamada et al., 1970; Hier et al., 1972). Hypothetically the effects of NGF and anti-NGF serum on the regenerating neuron might be regarded as an augmentation and diminution, respectively, of the retrograde response, which perhaps is normally influenced by the NGF (or NGF-like substance) present in the tissue and acting as a retrograde trophic influence on the neurons (cf. Hendry and

Iversen, 1973). The observations that the administration of NGF or anti-NGF serum at, or close to, the time of axonal damage resulted in the most pronounced effects on the regeneration process of central NA neurons (Papers V-VII) might further indicate that NGF is of particular importance during the very early stages or at the triggering of the sprouting process.

Apart from various central monoamine-containing neurons during regenerative growth appearing sensitive to NGF, it was recently found that in young adult, male mice NGF possibly plays a role also during regeneration of so-called short adrenergic neurons (Bjerre and Rosen-gren, 1974); a type of neurons previously not regarded as NGF target cells (cf. Levi-Montalcini, 1972). These findings contribute to further emphasise that the definition of an NGF target tissue remains open (cf. Introduction). It thus seems probable that future studies may reveal also other types of neural, and perhaps even non-neuronal (cf. Burnham et al., 1972), cell elements (for instance, in the CNS) as NGF target cells in the adult animal. This possibility could gain support from the present findings, suggesting that during an early stage of development perhaps all types of neural cells are dependent on NGF (or an NGF-like substance).

From those results indicating that NGF plays a role during the early development and differentiation of sympathoblasts, and also of other neural cell elements, it might be concluded that there is now evidence to contradict the idea that "NGF target cells become receptive to NGF only after they have acquired unmistakable differentiative marks" (Levi-Montalcini and Angeletti, 1968). It could thus rather be hypothesized that NGF is of importance, at least in the case of peripheral NA neurons, during the entire life of the animal, as there is no compelling evidence that such neurons at any stage, neither during the ontogenesis nor in the adult animal, should be non-receptive to NGF. However,

it could well be that the sensitivity to exogenous NGF is accentuated at critical stages, for instance during the early differentiation of the neurons and when the neurons start sprouting axons; i.e. at the time during ontogenesis when the outgrowth of axons normally occurs and after axonal damage in the adult animal, respectively. Such a possibility might also be interpreted in terms of a temporarily inadequate supply of endogenous NGF (or an NGF-like substance) at periods when the general performance of the neurons is speeded up. This would be a type of interpretation in accord with the ideas of Burnham et al. (1972) that sympathetic and sensory neurons are sensitive to exogenous NGF only in those situations where the support provided by surrounding, non-neuronal, tissue elements is inadequate. A role of NGF (or an NGF-like substance) in the interaction between neuronal and non-neuronal elements in the tissue would be a highly interesting possibility, which might imply that NGF (or an NGF-like substance) is a factor in the tissue which surrounds the neuron and is innervated by its axon terminals (cf. Hendry and Iversen, 1973) that controls the developmental events and the various performances of the neuron. Perhaps NGF thus could serve as a permissive factor, being a prerequisite for the mere survival of the neuron during the early differentiation and the ontogenetic development, and essential for much of the general performance of the neuron during later stages, i.e. for the maintenance of the axonal networks and for the regenerative growth of axons in the adult animal. Moreover, it could well be that NGF (or an NGF-like substance) — as a permissive factor — acts as a normal antagonist to other — restricting — factors in the regulation and termination of growth of axons and axon terminals during ontogenesis and also in the adult

animal. This idea is supported by recent, as yet unpublished, observations in our laboratory that exogenous NGF calls forth a collateral sprouting from intact NA axons in adult mice.

In this connection it would be interesting to investigate how a general decrease in the normal NGF levels in serum and tissues following salivectomy in mice might affect the general performance of peripheral NA neurons in the adult animal. This was partly studied by Hendry and Iversen (1973) who found a reduction in the superior cervical and the stellate ganglia in the activity of tyrosine hydroxylase, an enzyme involved in the synthesis of NA, and suggested that the NGF contained in the innervated end organs could act as a retrograde trophic influence on the neurons in the sympathetic ganglia. However, according to preliminary results the regenerative capacity of peripheral NA neurons does not appear markedly affected by the fall in NGF levels following salivectomy (Wiklund and Bjerre, in preparation). These somewhat contradictory findings seem to contribute to the general impression that much information is still required before the role of endogenous NGF is fully understood.

- Angeletti, P.U., Gandini-Attardi, D., Toschi, G.,
Salvi, M.L., and Levi-Montalcini, R.: Metabolic
aspects of the effect of nerve growth factor on
sympathetic and sensory ganglia in protein and
ribonucleic acid synthesis. *Biochim. biophys.*
Acta 95 (1965) 111-120.
- Angeletti, P.U., Liuzzi, A., and Levi-Montalcini, R.:
Stimulation of lipid biosynthesis in sympath-
etic and sensory ganglia by a specific nerve
growth factor. *Biochim. biophys. Acta* 84 (1964a)
778-781.
- Angeletti, P.U., Liuzzi, A., and Levi-Montalcini, R.:
Effects of nerve growth factor on glucose metab-
olism by sympathetic and sensory nerve cells.
Biochim. biophys. Acta 90 (1964b) 445-450.
- Angeletti, P.U., Levi-Montalcini, R., and Caramia, F.:
Ultrastructural changes in sympathetic neurons
of newborn and adult mice treated with nerve
growth factor. *J. Ultrastruct. Res.* 36 (1971a)
24-36.
- Angeletti, P.U., Levi-Montalcini, R., and Caramia, F.:
Analysis of the effects of the antiserum to
the nerve growth factor in adult mice. *Brain*
Res. 27 (1971b) 343-355.
- Angeletti, R.H., Angeletti, P.U., and Levi-Montalcini, R.:
Selective accumulation of [^{125}I] labelled nerve
growth factor in sympathetic ganglia. *Brain Res.*
46 (1972) 421-425.

- Angeletti, R.H. and Bradshaw, R.A.: Nerve growth factor from mouse submaxillary gland: Amino acid sequence. *Proc. Nat. Acad. Sci. (Wash.)* 68 (1971) 2417-2420.
- Banks, B.E., Banthorpe, D.U., Charlwood, K.A., Pearce, F.L., Vernon, C.A., and Edwards, D.C.: Distribution and immunology of mammalian nerve growth factor. *Nature* 246 (1973) 503-504.
- Bertler, Å., Carlsson, A., Rosengren, E., and Waldeck, B.: A method for the fluorimetric determinations of adrenaline, noradrenaline, and dopamine in tissues. *Kungl. Fysiogr. Sällsk. Lund Förh.* 28 (1958) 121-123.
- Bjerre, B.: The production of catecholamine-containing cells in vitro by young chick embryos studied by the histochemical fluorescence method. *J. Anat.* 115 (1973) 119-131.
- Bjerre, B. and Rosengren, E.: Effects of nerve growth factor and its antiserum on axonal regeneration of short adrenergic neurons in the male mouse. *Z. Zellforsch.* (1974, in press).
- Björklund, A., Bjerre, B., and Stenevi, U.: Has nerve growth factor a role in the regeneration of central and peripheral catecholamine neurons? In: K. Fuxe, L. Olson, and Y. Zotterman (Eds.), Dynamics of Degeneration and Growth in Neurons, Pergamon Press, Oxford, 1974 (in press).
- Björklund, A., Ehinger, B., and Falck, B.: A method for differentiating dopamine from noradrenaline in tissue sections by microspectrofluorometry. *J. Histochem. Cytochem.* 16 (1968) 262-270.

- Björklund, A., Ehinger, B., and Falck, B.: Analysis of fluorescence excitation peak ratios for the cellular identification of noradrenaline, dopamine, or their mixtures. *J. Histochem. Cytochem.* 20 (1972a) 56-64.
- Björklund, A., Falck, B., and Owman, Ch.: Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: J.E. Rall and I.J. Kopin (Eds.), Methods in Investigative and Diagnostic Endocrinology, Vol. I, The Thyroid and Biogenic Amines, North Holland Publ. Comp. Amsterdam, 1972b, pp. 318-368.
- Björklund, A., Falck, B., and Stenevi, U.: Classification of monoamine neurones in the rat mesencephalon: Distribution of a new monoamine neurone system. *Brain Res.* 32 (1971a) 1-17.
- Björklund, A., Katzman, R., Stenevi, U., and West, K.A.: Development and growth of axonal sprouts from noradrenaline and 5-hydroxytryptamine neurones in the rat spinal cord. *Brain Res.* 31 (1971b) 21-33.
- Björklund, A. and Stenevi, U.: Growth of central catecholamine neurones into smooth muscle grafts in the rat mesencephalon. *Brain Res.* 31 (1971) 1-20.
- Bocchini, V. and Angeletti, P.U.: The nerve growth factor: Purification as a 30,000-molecular-weight protein. *Proc. Nat. Acad. Sci. (Wash.)* 64 (1969) 787-794.
- Brattgård, S.-O., Edström, J.-E., and Hydén, H.: The chemical changes in regenerating neurons. *J. Neurochem.* 1 (1957) 316-325.

- Bueker, E.D.: Implantation of tumours in the hindlimb field of the embryonic chick and developmental response of the lumbosacral nervous system. *Anat. Rec.* 102 (1948) 369-390.
- Bueker, E.D., Schenkein, I., and Bane, J.L.: The problem of a nerve growth factor specific for spinal and sympathetic ganglia. *Cancer Res.* 20 (1960) 1220-1228.
- Burdman, J.A.: Early effects of a nerve growth factor on the RNA content and base ratios of isolated chick embryo sensory ganglia neuroblasts in tissue culture. *J. Neurochem.* 14 (1967) 367-371.
- Burdman, J.A. and Goldstein, M.N.: Synthesis and storage of a nerve growth protein in mouse submandibular glands. *J. Exp. Zool.* 160 (1965) 183-188.
- Burnham, P., Raiborn, C., and Varon, S.: Replacement of nerve growth factor by ganglionic non-neuronal cells for the survival in vitro of dissociated ganglionic neurons. *Proc. Nat. Acad. Sci. (Wash.)* 69 (1972) 3556-3660.
- Burnham, P. and Varon, S.: In vitro uptake of a active nerve growth factor by dorsal root ganglia of embryonic chick. *Neurobiology* 3 (1973) 232-245.
- Cajal, S.R.Y.: Degeneration and Regeneration of the Nervous System. Vol. 2 (translated and edited by R.M. May), Oxford University Press, London, 1928.
- Clemente, C.D.: Regeneration in the vertebrate central nervous system. *Int. Rev. Neurobiol.* 6 (1964) 257-301.
- Cohen, A.M.: Factors directing the expression of sympathetic nerve traits in cells of neural crest origin. *J. Exp. Zool.* 179 (1972) 167-182.

- Daniels, M.P.J.: Abstract. J. Cell Biol. 39 (1968).
- De Champplain, J.: Degeneration and regrowth of adrenergic nerve fibres in the rat peripheral tissues after 6-hydroxydopamine. Can. J. Physiol. Pharmac. 49 (1971) 345-355.
- Enemar, A., Falck, B., and Håkansson, R.: Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo. Devel. Biol. 11 (1965) 268-283.
- Falck, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta Physiol. Scand. 56, Suppl. 197 (1962) 1-91.
- Falck, B., Hillarp, N.-Å., Thieme, G., and Torp, A.: Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 10 (1962) 348-354.
- Fischer, S. and Jacobson, M.: Ultrastructural changes during early development of retinal ganglion cells in *Xenopus*. Z. Zellforsch. 104 (1970) 165-177.
- Gallera, J. and Ivanov, I.: La compétence neurogène du feuillet externe du blastoderme de Poulet en fonction du facteur "temps". J. Embryol. exp. Morph. 12 (1964) 693-711.
- Gallera, J., Nicolet, G., and Baumann, M.: Induction neurale chez les Oiseaux à travers un filtre millipore: étude au microscope optique et électronique. J. Embryol. exp. Morph. 19 (1968) 439-450.
- Haeusler, G., Haefely, W., and Thoenen, H.: Chemical sympathectomy of the cat with 6-hydroxydopamine. J. Pharmacol. exp. Ther. 170 (1969) 50-61.

- Häggendal, J.: An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *Acta Physiol. Scand.* 59 (1963) 242-254.
- Halstead, D.C. and Larrabee, M.G.: Early effects of anti-serum to the nerve growth factor on metabolism and transmission in superior cervical ganglia of mice. In: G. Steiner and E. Schönbaum (Eds.), Immunosympathectomy, North Holland Publ. Comp., Amsterdam, 1972, pp. 221-236.
- Hamburger, V. and Hamilton, H.L.: A series of normal stages in the development of the chick embryo. *J. Morph.* 88 (1951) 49-92.
- Hendry, I.A.: Development changes in tissue and plasma concentrations of the biologically active species of nerve growth factor in the mouse, by using a two-site radioimmunoassay. *Biochem. J.* 128 (1972) 1265-1272.
- Hendry, I.A., Addison, G.M., and Iversen, L.L.: Radioimmunoassay of nerve growth factor from mouse salivary gland. In: E. Zaimis and J. Knight (Eds.), Nerve Growth Factor and its Antiserum, Athlone Press, London, 1972, pp. 262-270.
- Hendry, I.A. and Iversen, L.L.: Changes in tissue and plasma concentrations of nerve growth factor following removal of the submaxillary glands in adult mice and their effects on the sympathetic nervous system. *Nature* 243 (1973) 500-504.
- Hendry, I.A., Stöckel, K., Thoenen, H., and Iversen, L.L.: The retrograde axonal transport of nerve growth factor. *Brain Res.* 68 (1974) 103-121.

- Hier, D.B., Arnason, B.G.W., and Young, M.: Studies on the mechanism of action of nerve growth factor. Proc. Nat. Acad. Sci. (Wash.) 69 (1972) 2268-2272.
- Hillman, H. and Sheikh, K.: The growth in vitro of new processes from vestibular neurons isolated from adult and young rabbits. Exp. Cell Res. 50 (1968) 315-322.
- Hunt, T.E.: Hensen's node as an organizer in the formation of the chick embryo. Abstract. Anat. Rec. 42 (1929) 22.
- Hunt, T.E.: An experimental study of the independent differentiation of the isolated Hensen's node and its relation to the formation of axial and non-axial parts in the chick embryo. J. Exp. Zool. 59 (1931) 395-427.
- Jacobson, M.: Developmental Neurobiology, Holt, Rinehart, and Winston, New York, 1970, pp. 213-223.
- Johnson, D.G., Gordon, P., and Kopin, I.J.: A sensitive radioimmunoassay for 7 S nerve growth factor antigens in serum and tissues. J. Neurochem. 18 (1971) 2355-2362.
- Johnson, D.G., Silberstein, S.D., Hanbauer, I., and Kopin, I.J.: The role of nerve growth factor in the ramification of sympathetic nerve fibres into the rat iris in organ culture. J. Neurochem. 19 (1972) 2025-2029.
- Jonsson, G. and Sachs, Ch.: Neurochemical properties of adrenergic nerves regenerated after 6-hydroxydopamine. J. Neurochem. 19 (1972) 2577-2585.
- Källén, B.: Early morphogenesis and pattern formation in the central nervous system. In: R.L. De Haan and H. Ursprung (Eds.), Organogenesis, Holt, Rinehart, and Winston, New York, 1965, pp. 107-128.

- Källén, B.: Early embryogenesis of the central nervous system with special reference to closure defects. *Develop. Med. Child. Neurol. Suppl.* 16 (1968) 44-53.
- Kapeller, K. and Mayor, D.: An electron microscopic study of the early changes proximal to a constriction in sympathetic nerves. *Proc. Roy. Soc. (London), Ser. B*, 172 (1969) 35-51.
- Kumar, S. and Steward, J.K.: A possible site of action of the nerve growth factor. *Exp. Cell Res.* 92 (1970) 200-201.
- Larrabee, M.G.: Metabolism during development in sympathetic ganglia of chickens: Effects of age, nerve growth factor, and metabolic inhibitors. In: E. Zaimis and J. Knight (Eds.), Nerve Growth Factor and its Antiserum, Athlone Press, London, 1972, pp. 71-88.
- Leikola, A. and McCallion, D.J.: Time required for heterogenous induction in chick embryo ectoderm. *Experientia* 23 (1967) 869-870.
- Leikola, A. and McCallion, D.J.: Inductive capacity of killed Hensen's node and head process in the chick embryo. *Can. J. Zool.* 46 (1968) 205-207.
- Levi-Montalcini, R.: Effects of mouse tumor transplantation on the nervous system. *Ann. N.Y. Acad. Sci.* 55 (1952) 330-343.
- Levi-Montalcini, R.: Chemical stimulation of nerve growth. In: W.D. McElroy and B. Glass (Eds.), Chemical Basis of Development, John Hopkins Press, Baltimore, 1958, pp. 646-664.
- Levi-Montalcini, R.: The nerve growth factor: Its mode of action on sensory and sympathetic nerve cells. *Harvey Lectures Ser.* 60 (1966) 217-259.

- Levi-Montalcini, R.: The morphological effects of immunosympathectomy. In: G. Steiner and E. Schönbaum (Eds.), Immunosympathectomy, North Holland Publ. Comp., Amsterdam, 1972, pp. 55-78.
- Levi-Montalcini, R. and Angeletti, P.U.: Immunosympathectomy. *Pharmacol. Rev.* 18 (1966) 619-628.
- Levi-Montalcini, R. and Angeletti, P.U.: Nerve growth factor. *Physiol. Rev.* 48 (1968) 534-569.
- Levi-Montalcini, R. and Angeletti, P.U.: Nerve growth factor. Evaluation and perspectives. In: E. Zaimis and J. Knight (Eds.), Nerve Growth Factor and its Antiserum, Athlone Press, London, 1972, pp. 46-58.
- Levi-Montalcini, R., Angeletti, R.H., and Angeletti, P.U.: The nerve growth factor. In: G.H. Bourne (Ed.), The Structure and Function of Nervous Tissue, Vol. V, Academic Press, New York and London, 1972, pp. 1-38.
- Levi-Montalcini, R. and Booker, B.: Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve-growth promoting factor. *Proc. Nat. Acad. Sci. (Wash.)* 46 (1960) 384-391.
- Levi-Montalcini, R., Caramia, F., Luse, S.A., and Angeletti, P.U.: In vitro effects of the nerve growth factor on the fine structure of the sensory nerve cells. *Brain Res.* 8 (1968) 347-362.
- Levi-Montalcini, R. and Cohen, S.: In vitro and in vivo effects of a nerve growth-stimulating agent isolated from snake venom. *Proc. Nat. Acad. Sci. (Wash.)* 42 (1956) 695-699.

- Levi-Montalcini, R. and Cohen, S.: Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic anti-serum. Proc. Nat. Acad. Sci. (Wash.) 46 (1960) 302-311.
- Levi-Montalcini, R. and Hamburger, V.: Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool. 116 (1951) 321-362.
- Levi-Montalcini, R., Meyer, H., and Hamburger, V.: In vitro experiments on the effects of mouse sarcoma 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. 14 (1954) 49-57.
- Lyser, K.M.: The development of the chick embryo diencephalon and mesencephalon during the initial phases of neuroblast differentiation. J. Embryol. exp. Morph. 16 (1966) 497-517.
- Matthews, M.R. and Raisman, G.: A light and electron microscopic study of the cellular response to axonal injury in the superior cervical ganglion of the rat. Proc. Roy. Soc. (London), Ser. B, 181 (1972) 43-79.
- Nord, L.: Immunofluorescence studies on the development of early mouse and chick nervous system in vivo and in vitro. Thesis, University of Lund, 1969.
- Norr, S.C.: In vitro analysis of sympathetic neuron differentiation from chick neural crest cells. Devel. Biol. 34 (1973) 16-38.
- Olson, L.: Outgrowth of sympathetic adrenergic neurons in mice treated with a nerve-growth factor (NGF). Z. Zellforsch. 81 (1967) 155-173.

- Olson, L. and Seiger, Å.: Brain tissue transplanted to the anterior chamber of the eye. I. Fluorescence histochemistry of immature catecholamine and 5-hydroxytryptamine neurons re-innervating the rat iris. *Z. Zellforsch.* 135 (1972) 175-194.
- Partlow, L.M. and Larrabee, M.G.: Effects of a nerve-growth factor, embryo age and metabolic inhibitors on growth of fibres and on synthesis of ribonucleic acid and protein in embryonic sympathetic ganglia. *J. Neurochem.* 18 (1971) 2101-2118.
- Pasternak, L. and McCallion, D.J.: Heterogenous inductions in the chick embryo. *Can. J. Zool.* 40 (1962) 585-591.
- Pérez-Polo, J.R., Bamburg, J.R., de Jong, W.W.W., Straus, D., Baker, M., and Shooter, E.M.: Nerve growth factors of the mouse submaxillary gland. In: E. Zaimis and J. Knight (Eds.), Nerve Growth Factor and its Antiserum, Athlone Press, London, 1972, pp. 19-34.
- Roisen, F.J. and Murphy, R.A.: Neurite development in vitro: II. The role of microfilaments and microtubules in dibutyryl adenosine 3',5'-cyclic monophosphate and nerve growth factor stimulated maturation. *J. Neurobiol.* 4 (1973) 397-412.
- Rostedt, I.: A study of the action of heterogenous inducers on chicken embryo ectoderm. *Scand. J. Clin. Lab. Invest.* 21, Suppl. 101 (1968) 49.
- Sabatini, M.T., Pellegrino de Iraldi, A., and de Robertis, E.: Early effects of antiserum against the nerve growth factor on fine structure of sympathetic neurons. *Exp. Neurol.* 12 (1965) 370-383.

- Saunders, N.R.: Lack of effect of nerve growth factor on peripheral sensory nerve regeneration. In: E. Zaimis and J. Knight (Eds.), Nerve Growth Factor and its Antiserum, Athlone Press, London, 1972, pp. 116-122.
- Saxén, L. and Toivonen, S.: Primary embryonic induction, Logos Press, Academic Press, London, 1962.
- Scott, D. and Liu, C.N.: Factors promoting regeneration of spinal neurons: Positive influence of nerve growth factor. In: M. Singer and J.P. Schadé (Eds.), Mechanisms of Neural Regeneration, Progress in Brain Research, Vol. 13, Elsevier Publ. Comp., Amsterdam, 1964, pp. 127-150.
- Silberstein, S.D., Johnson, D.G., Jacobowitz, D.M., and Kopin, I.J.: Sympathetic reinnervation of the rat iris in organ culture. Proc. Nat. Acad. Sci. (Wash.) 68 (1971) 1121-1124.
- Smith, A.P., Varon, S., and Shooter, E.M.: Multiple forms of the nerve growth factor protein and its subunits. Biochemistry (Wash.) 6 (1967) 2202-2209.
- Svendgaard, N.-A., Bjerre, B., and Björklund, A.: In preparation.
- Toivonen, S. and Saxén, L.: Morphogenetic interaction of presumptive neural and mesodermal cells mixed in different ratios. Science 159 (1968) 539-540.
- Tranzer, J.P. and Richards, J.G.: Fine structural aspects of the effect of 6-hydroxydopamine on peripheral adrenergic neurons. In: T. Malmfors and H. Thoenen (Eds.), 6-Hydroxydopamine and Catecholamine Neurons, North-Holland Publ. Comp., Amsterdam-London, 1971, pp. 15-31.

- Ungerstedt, U.: Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol. Scand.*, Suppl. 367 (1971) 1-48.
- Varon, S., Nomura, J., and Shooter, E.M.: The isolation of the mouse nerve growth factor protein in a high molecular weight form. *Biochemistry (Wash.)* 6 (1967a) 2202-2209.
- Varon, S., Nomura, J., and Shooter, E.M.: Subunit structure of a high-molecular-weight form of the nerve growth factor from mouse submaxillary gland. *Proc. Nat. Acad. Sci. (Wash.)* 57 (1967b) 1782-1789.
- Waddington, C.H.: Experiments on the development of chick and duck embryos, cultivated in vitro. *Phil. Trans. B* 221 (1932) 179-230.
- Waddington, C.H.: Experiments on embryonic induction. I-III. *J. Exp. Biol.* 11 (1934) 211-277.
- Waddington, C.H.: The Epigenetics of Birds, University Press, Cambridge, 1952.
- Watson, W.E.: Some metabolic responses of axotomized neurones to contact between their axons and denervated muscle. *J. Physiol.* 210 (1970) 321-343.
- Weis, P. and Bueker, E.D.: Ligation of mouse submandibular glands and its effects on components of nerve growth factor. *Proc. Soc. Exp. Biol. Med.* 121 (1966) 1135-1140.
- Weston, J.A.: The migration and differentiation of neural crest cells. In: M. Abercrombie, J. Brachet, and T. King (Eds.), Advances in Morphogenesis, Vol. 8, Academic Press, New York and London, 1970, pp. 41-114.

- Wetzel, R.: Untersuchungen am Hünchen. Die Entwicklung des Keims während der ersten beiden Bruttage. Wilhelm Roux' Arch. Entwickl.-Mech. Org. 119 (1929) 188-321.
- Wiklund, L. and Bjerre, B.: In preparation.
- Willier, B.H. and Rawles, M.E.: The relation of Hensen's node to the differentiating capacity of whole chick blastoderms as studied in chorioallantoic grafts. J. Exp. Zool. 59 (1931) 429-465.
- Windle, W.F.: Regeneration in the Central Nervous System, C.C. Thomas, Springfield, U.S.A., 1955.
- Winick, M. and Greenberg, R.E.: Appearance and localization of a nerve growth-promoting protein during development. Pediatrics 35 (1965) 221-228.
- Woodside, G.L.: The influence of host age on induction in the chick blastoderm. J. Exp. Zool. 75 (1937) 259-277.
- Yamada, K.M., Spooner, B.S., and Wessels, N.K.: Axon growth: Roles of microfilaments and microtubules. Proc. Nat. Acad. Sci. (Wash.) 66 (1970) 1206-1212.
- Yamada, K.M. and Wessels, N.K.: Axon elongation. Effect of nerve growth factor on microtubule protein. Exp. Cell Res. 66 (1971) 346-352.

